Pakistan J. Zool., vol. 50(1), pp 381-384, 2018. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2018.50.1.sc5

Short Communication

Identification of Bacteria from Diarrheic foals in Punjab, Pakistan

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ABSTRACT

Foal diarrhea in working equines is reported as the most common cause of mortality. Lahore and Sahiwal districts of Punjab, Pakistan were selected during 2016 for the present study. A total of 616 foals were examined out of which 447 diarrheic foals were sampled for isolation of bacteria. During the study 294 faecal samples (horse n=217, mules n=23 and donkey n=54) were diagnosed positive for bacteria. The overall prevalence of diarrhea was 72.56% with bacteria in 65.77% of diarrheic cases. The prevalence of different bacterial species shows that *E. coli* was the most prevalent bacteria with 48.77% as compared to *Clostridium perfringens* (18.56%) and *Salmonella* (17.9%). It was concluded that bacteria were the major cause of foal diarrhea in Punjab, Pakistan.

Article Information
Received 12 July 2017
Revised 02 September 2017
Accepted 07 November 2017
Available online 03 January 2018

Authors' Contributions
AZD, MSK, MHM and IH designed the study. IH and AZD executed the experiment and analysed the samples. IA, AK and MA helped in data analysis and article drafting.

Key words
Foal diarrhea, *Salmonella, Clostridium perfringens, E. coli*

Almost 80% of the foals, experience at least one episode of diarrhea in their life (Frederick *et al*., 2009). The causes of diarrhea may be infectious or non-infectious. The infectious causes of diarrhea include *Salmonella* Spp., *Escherichia coli*, *Clostridium perfringens* and Rotavirus (Lester and Magdigen, 2009). Among bacterial causes of foal diarrhea Salmonellosis is considered as an important and deadly disease caused by different serotypes of *Salmonella enterica* as compared to any other bacterial infection (Hassenin *et al*., 2010a). *Salmonella* is reported to be more prevalent in astrointestinal ailments in foals (Hartmann *et al*., 1996). Along with *Salmonella*, *E. coli* is most commonly isolated bacterium from foals (Hassenin *et al*., 2010b). The other species of bacteria involved in the disease are *Clostridium* (Netherwood *et al*., 1996). Salmonellosis occurs in different clinical forms in horses resulting in severe diarrhea and fatal septicemia in foals (Kohn, 1982). It causes higher mortality as compared to any other bacterial infection. Studies have shown 13% prevalence of Salmonellosis in foals with gastrointestinal disease. The recovered animals may shed the organism for variable period of time. The infection occurs through contaminated food and water (Hartmann *et al*., 1996) .In India *Salmonella* were isolated from 6.77% of faecal samples of equines (Singh *et al*., 2007). *S. typhimurium* was found as common bacteria in horses (Rajasekhar and Babu, 1992). Different strains of entero-toxigenic *E. coli* were isolated from foals (Holland *et al*., 1996). The bacterium is considered virulent due to its ability of affecting epithelium of intestinal tract (Mainil *et al*., 1993; Fisher *et al*., 1994). *E. coli* produce enterotoxins that cause epithelial cells to secrete electrolytes and water profusely and results in diarrhea (Ooms and Degryse, 1986). Sporadic cases of diarrhea in foals were reported caused by different type of *Clostridium* (Netherwood *et al*., 1996). Isolation and identification of bacteria causing foal diarrhea is important as some bacteria are zoonotic (Tillotson *et al*., 1997). Data on prevalence of foal diarrhea caused by different types of bacteria is not available in Pakistan. This is the first study of its kind in the country because an active surveillance is prerequisite to determine the extent of bacterial causes of foal mortality.

Material and methods

The study was carried out in rural and urban areas of Lahore and Sahiwal districts of Punjab, Pakistan to record the prevalence of bacterial diarrhea in foals during year 2016.

A total of 447 freshly passed faecal samples (foals up to 1 year of age) were collected from diarrheic foals of different species (horse n=318; donkey n=82 and mule n=47) in sterilized labeled container having phosphate buffered saline. The samples were cultured for isolation of Salmonella, *C. perfringens* and *E. coli* with subsequent PCR for confirmation of microbiota involved.

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0030-9923/2018/0001-0381 $ 9.00/0

All 447 faecal samples were extended in 10 ml buffered peptone water and incubated at 37°C for 18 h. After pre-enrichment, faecal samples were selectively enriched in tetrathionate broth (TTB) at 42°C for 24 h for salmonella. For *C. perfringens*, samples were enriched in anaerobically sterilized Brucella broth and for *E. coli* were enriched in brain-heart infusion (BHI). After 24 h, a loopful was taken from the enriched samples and were streaked on brilliant green agar plates and the cultured plates were incubated at 37 °C for 24 h for salmonella and *E. coli* (Singh *et al*., 2007). *Clostridium perfringens* agar base medium supplemented with egg yolk emulsion @ 50 ml/ liter and D-cycloserine @ 2 vials/litter was used to culture *C. perfringens* (Despina *et al*., 2010). Suspected colonies of *Salmonella* (transparent colonies with reddish periphery), *E. coli* (colorless) and *C. perfringens* (black color) were re-cultured to get pure culture (Singh *et al*., 2007).

DNA was extracted using commercially available DNA extraction Kit (QIAGEN, Germany, Qiagen Street 1, 40724 Hilden, Germany) accordinag to company guidelines and procedure.

Data from questionnaires were entered into Microsoft Excel spread sheet. Data was validated through crosschecking the computerized recorded data with those of original complete data copy. Statistical analysis of data was conducted through SPSS Version 20.00. Prevalence data were analyzed by applying Chi-square test.

Results

Salmonella colonies appeared as transparent colonies with reddish periphery, on brilliant green agar. The suspected colonies were re-cultured for purification and expressed same colony characteristics. *E. coli* showed yellowish green colonies on brilliant green agar and arepeated same characteristics after re-culture. *C. perfringens* showed black colonies surrounded by transparent zones in cultures in *C. perfringens* agar base medium supplemented with egg yolk emulsion @ 50 ml/ liter and D-Cycloserine @ 2 vials/ litters.

Using the genus specific primer, a product of 496bp was generated for *Salmonella* species. The bacteria were found involved in the disease independently in 36 horse foals and in 10 donkey foals (Table I). The bacteria were not found in mule foals as the only cause of infection. However, the bacterium was found in mixed bacterial infection in all three species of equines (Table I). A product of 405bp was generated in case of *C. perfringens*. The bacteria were found in 11 horse and 4 donkey foals. In mules, the bacterium was involved in mixed infections. The *E. coli* infection was conformed in 112 horses, 9 mules and 32 donkey foals as a sole cause of bacterial diarrhea (Table I). A 200bp product was generated in PCR.

An overall prevalence of foal diarrhea in two geographical locations of Punjab was 72.56%. The overall bacterial cause of diarrhea was found 65.77% among diarrheal foals. The prevalence of diarrhea in horse, mule and donkey foals was 72.93, 66.19 and 75.22% respectively (Table II). The bacterial diarrhea in three species revealed 68.55, 48.93 and 65.85% disease, respectively. Data on gender (in case of mule the appearance of the foal was considered) revealed 68.91% in male and 75.93% in female foals. The bacterial infection was found 58.82% in male 72.01% in female among diarrheal animals. Area wise prevalence was 72.58 and 72.54% in Lahore and Sahiwal districts. The bacteria were isolated from 45.48 and 50% of the diarrheal animals. Chi-square analysis showed significant difference ($p<0.05$) between male and female animals in overall disease in foals. The data on bacterial foal diarrhea revealed significant difference among species, gender and area (Table II). The bacteria were found in 294 animals as mixed and single infection. The prevalence of different bacterial species show that *E. coli* was the most prevalent bacteria with 48.77% as compared to *C. perfringens* (18.56%) and Salmonella (17.9%) (Table II). The bacteria were isolated as infection with single specie or mixed infection with other bacteria. The data showed that 80 foals were infected with 2 or more than 2 bacteria. Chi-square analysis showed significant difference ($p<0.05$) except area wise prevalence of single or mixed bacterial infection (Table I).

Discussion

Foal diarrhea was the major reported cause of mortality in early age (Baverud *et al*., 2003). The diagnosis was made through different techniques. The culture of bacteria and subsequent confirmation through PCR was performed by many researchers (Singh *et al*., 2007; Walker *et al*., 1995) amplification of DNA (PCR) was reported as efficient method of confirmation (Amavisit *et al*., 2001; Ward *et al*., 2005). The prevalence rate of 80% diarrhea up to six months (age) was reported by (Frederick *et al*., 2009; Dunkel and Wilkins, 2004). The findings of the present study were in line with these reports. The findings of the present study were in agreement with previous Reported studies that showed that the major bacterial species involved in foal diarrhea were Salmonella species (Fredrick *et al*., 2009), *C. perfringens* (Netherwood *et al*., 1996; Weese *et al*., 2001; Tillotson *et al*., 2002; Fredrick *et al*., 2009) and *E. coli* (Hamzah *et al*., 2013; Quinn *et al*., 2002). These three species were found in foals of horse, mule and donkey in both areas of Punjab. In the present study, *E. coli* was found the most prevalent specie of bacteria. The findings are in agreement with earlier study conducted by Hamzah *et al*. (2013) who reported *E. coli* 48.75% and 44.00%, respectively. The higher prevalence of the *E. coli* may due to the presence of the bacteria in equine gastrointestinal tract as commensal organism Quinn *et al*. (2002). The prevalence

Table I.- Species of bacteria isolated from Diarrheic foals in different district of Punjab, Pakistan.

Variable	District Lahore	District Sahiwal	Total	p- value
No. of diarrheic foals with mixed bacterial infection	33	47	80	
Positive only for salmonella spp	25	21	46	
Positive only for only for *C. perfringens*	7	8	14	
Positive only for *E. coli*	76	77	153	
Total	141	153	294	0.472

Table II.- Prevalence of bacteria in Diarrheic foals in Lahore and Sahiwal districts of Punjab, Pakistan.

Variables		No. of diarrheic foals	No. of foals with bacterial infection	Prevalence% of bacterial diarrhea	p-value
Species	Horse	318	217	68.23	
	Mule	47	23	48.93	0.05
	Donkey	82	54	65.85	
Gender	Male	204	120	58.82	0.03
	Female	243	174	71.16	
Districts	Lahore	225	141	45.48	0.09
	Sahiwal	222	153	50	

of *E. coli* in male foals was 47.05 % while in female foal it was isolated from 50.02% of foals. Same trend was shown by the study conducted by Harris *et al*. (2012). In our study the prevalence of *E. coli* was more in horses (49.37%) as compared to mules (48.9%) and donkey foals (46.34%). Quinn *et al*. (2002), Hamzah *et al*. (2013) reported similar findings. In the present study, *Salmonella* was isolated from 17.9% of the samples. Our findings are in line with the earlier studies in which salmonella was documented as important cause of equine diarrhea, Singh *et al*. (2007) reported 13% and Fredrick *et al*. (2009) 12% *Salmonella* in foals. The study conducted in India having similar geographic conditions showed 6% prevalence of salmonella in equines using culture technique and 12.5% with PCR (Singh *et al*., 2007). The study also agrees with earlier report that showed 1% to 17% prevalence of salmonella from faecal sample or rectal swabs (Traub-Dargatz et al., 2000; Babu, 2003). The results showed that the occurrences of salmonella were significantly associated with the gender of the foals. The prevalence was high in females (20.1%) as compared to males (14.21%). Our results were not with in agreement with pervious study conducted by Harris *et al*. (2012). Species wise prevalence of salmonella in our study showed higher percentage in horses than donkeys. The results are concurrence with earlier study conducted by (Singh *et al*., 2007). In the present study, *C. Perfringens* was isolated and confirmed from 18.56% of the diarrheic foals. The results of the study were in agreement with the pervious study by Fredrick *et al*. (2009). The bacteria were found significantly involved in foal diarrhea (Netherwood *et al*. 1996). Tillotson *et al*. (2002) reported that 85% of isolates were type A and were found positive for entero toximic genes. The study is not in agreement with previous study in which *C. perfringens* was isolated from 90% of foals from 6 farms and reported non-significant association between the isolation of bacteria and the occurrences of diarrhea (Tillotson *et al*., 2002). The result showed that prevalence of *Clostridium Perfirengenes* was 18.8% in females and was 16.86% in male foals. The results of our study showed same trend as reported by earlier study conducted by Harris *et al*. (2012). The prevalence of *C. perfringens* in different species of equines showed that the prevalence was high in horses (18.86%) as compared to donkeys (14.74%). Earlier reports showed 18% prevalence of *C. perfringens* in horses (Fredrick *et al*., 2009) and 90% in diarrheic horse foals (Tillotson *et al*., 2002). The bacterial diarrhea in female foals was significantly higher than males. Contrarily Singh *et al*. (2007) reported more bacterial infection in male foals. This difference in findings might be due to the difference in management or sample size of the two studies. The difference between male and female foals may be attributed to the physiology and stress difference of gender (Carter *et al*., 1986). The disease was reported significantly high in mules followed by horses and donkeys (Singh *et al*., 2007; Singh *et al*., 2005) due to higher susceptibility of intestine to colonization of bacteria due to exposure to unhygienic conditions in mules. Losinger *et al*. (2002) also reported more prevalence in mule with the view that mules are grouped for draught purpose. These studies were reported in adult animals. The data of the present study revealed higher prevalence in horse foals (68.23%) followed by donkeys (65.85%) and mules (48.93%). The difference may attribute to the age and number of samples. During the present study, higher prevalence of bacterial diarrhea was found in Sahiwal district (50%). The trend of the disease was found almost similar between two regions.

It can be concluded that isolation of *E. coli*, *Salmonella* and *C. perfringens* from foals of Lahore and Sahiwal districts of Punjab, Pakistan indicated that

the bacteria are prevalent in these districts as intestinal microbiota since early ages. Further investigation from different geographical region of country are pre-requisite to equines foals.

Acknowledgement

The authors are highly obliged to Higher Education Commission (HEC) Pakistan for funding the research under Project No. 4263; Titled: "Epidemiology of Major Viral, Bacterial and Parasitic Causes of Foal Diarrhea" The authors also appreciate the cooperation of RVFC in field work of the project.

Statement of conflict of interest

Authors have declared no conflict of interest.

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